24.8.2015

MONDAY, 8/24

Petra

Checking transformation plates from friday:

- CAR in pSB1C3: no colonies except one very small colony in CAR 4 #1 plate
- CAR in AH043: lots of colonies in every plate

Making o/n cultures for SDS-PAGE
Chose one colony from CAR 4 (in pSB1C3) #1 plate and one colony from each CAR in AH043 backbone (CAR 2, CAR 3, CAR 4 and CAR 7). Made o/n culture of non-transformed DEL1 cells for control.

- 2 ml LB
- 2 ul antibiotic stock (amp for AH043 backbone, cam for pSB1C3, kan for DEL1 cells)

Left the tubes to 37 C incubator (with shaking) overnight.

Did restrictions of AH045 with E & S following mostly the protocol. However, the incubation was 1,5 h.

Ligated twice the restrictions of AH045 with pSB1K3 (19.8.) and GFP c3/c4 (19.8.). One set of ligations was done following the T4 ligase protocol's suggestion of molar ratio of inserts (GFP c3/c4 ; AH045) and a vector (pSB1K3). The other set was done so that the volume of an insert was same as the volume of the vector (GFP c3/c4 2 ul; AH045 2 ul; pSB1K3 2 ul).

Transformed the ligations to Top10 CHEM competent cells following the protocol. Plated 50 ul and 150 ul from each tube (total: 4 tubes) to KAN plates. Incubated in 37 C o/n.

25.8.2015

TUESDAY, 8/25

Petra

Preparing cultures for SDS-PAGE:
Refreshed yesterday's o/n cultures: 100 ul o/n culture to 2 ml fresh LB with appropriate antibiotics. Incubated in 37 C with skaking until OD reached 0,5 (~1h 20min). Added 0,5 ul IPTG (final IPTG concentration in the cultures: 0,5 mM). Started incubating in 37 C with shaking at 10.50. Incubated the samples until 13.30.

Preparing samples for SDS-PAGE
Followed the protocol. Stored the samples to the freezer o/n.

Decided to try Gibson assembly for Cellulose and AtoB constructs with linearized pSB1C3 backbone that has been restricted with EcoRI and PstI.
Restricted 500 ng (20 ul) of linearized pSB1C3. Used NeBuffer 3.1 for the reaction. Added 0,7 ul EcoRI and 0,5 ul Pst1. Did'n add any water. Total volume of the reaction: 23,7 ul. Incubated in 37 C for 1 h 45 min. When deactivating restriction reaction in 80 C the sample was forgotten to heatblock. Deactivation lasted for 45 minutes (20 min recommended).

Gibson assembly for AtoB:
Pipetted DNA according to Table 1.

Table1

Construct	Length (bp)	Concentration (ng/ul)	DNA (ng) needed for 0.05 pmol	DNA (ul) needed for 0,05 pmol
AtoB part 1	1311	106,1	40,51	0,38
fixed AtoB part 2	2000	278,5	61,8	0,22
AtoB part 3	1772	284,3	54,75	0,2
AtoB part 4	1414	52,1	43,69	0,84
restricted pSB1C3	2070	21,1	63,96	3,0
Total:				4,64

Added 4,64 ul MasterMix. Didn't add any water. Incubated in 50 C for 1 h. Stored the mix to the freezer to transform tomorrow.

Gibson assembly for CenA:
Pipetted DNA according to Table 2.

Table2

Construct	Lenght	Concentration (ng/ul)	DNA (ng) needed for 0.05 pmol	DNA (ul) needed for 0,05 pmol
CenA part 1	1891	122	58,43	0,48
CenA part 2	1302	114,3	40,23	0,35
BglX	2440	345,9	75,39	0,22
restrcted pSB1C3	2070	21,1	63,96	3,0
Total:				4,05

Added 4,05 ul MasterMix. Didn't add any water. Incubated in 50 C for 1 h. Stored the mix to the freezer to transform tomorrow.

Casting the SDS-PAGE gel:
Followed the protocol. Made a 18 % separating gel.

Even though yesterday's transformation of AH045+GFP was successful, did restriction, ligation and transformation again, because any colony in the plate didn't glow in UV (even tough thre is GFP) and because of sequening results tried to use a different GFP colony (GFP c4 to GFP c2).

Did restrictions of AH045 with E & S, pSB1K3 with E & P and GFP c2 and GFP c3 with X & P following mostly the protocol. However, the incubation was 1,5 h.

Ligated twice the restrictions of AH045 with pSB1K3 and GFP c2/c3. One set of ligations was done following the T4 ligase protocol's suggestion of molar ratio of inserts (GFP c2/c3 3,2 ul; AH045 0,3) and a vector (pSB1K3 3 ul). The other set was done so that the volume of an insert was higher than the volume of the vector (GFP c2/c3 3 ul; AH045 4 ul; pSB1K3 2 ul).

Transformed the ligations to Top10 CHEM competent cells following the protocol. Plated 50 ul and 150 ul from each tube (total: 4 tubes) to KAN plates. Incubated in 37 C o/n.

Did o/n cultures of yesterday's successful transformants (AH045+pSB1K3+GFP c3/c4; colonies on the plate) in 2 ml LB with 2 ul KAN.

Juuso

for Petra:
16:30 Pulled out the combs from 2 SDS-PA-gels prepared by Petra, wrapped the gels in wet paper and put both gelsin a minigrip bag. Marked the bag "2 X SDS-PAGE GEL 25.8.15 JR -> PT" and stored at +4C (box "heli").

18:00 Took two Petras tubes from 50C heat block: "AtoB gibson 25.8." and "CenA gibson 25.8". Taped them together with orange tape, wrote "PETRA 25.8. 18:00 50C lämpöblokista" on the tape and stored in -20C iGEM box.

for Tamannae:
16:00 Pipetted 2 µl kanamycin solution per tube (each containing 2 ml medium) to 17 tubes altogether (marked 1-17)

16:15 Plated transformants from tubes trans C2, C2 II, C3 and C3 II to kanamycin plates. 50 µl and 150 µl of each transformant were plated on separate plates, 8 plates altogether.

Marked plates "kan C[2/3] [II] [50/150] µl 25.8.15 JR->TA" and let them dry until 16:40, after which I placed them in +37C (gene lab incubator)

17:20 Inoculated colonies circled and numbered 1-17 by Tamannae from C4 plates to 2 ml kanamycin tubes with respective numbers, in total 17 tubes/colonies. Placed tubes in +37C incubator in gene lab, in the shaker.

for Purpald test:

16:50 Took Petras 1ml tubes from +37C shaking and pipeted contents to 2 ml eppendorf tubes named:

- "CAR7 in AH043" (was in amp)
- "CAR4 in AH043" (was in amp)
- "CAR2 in AH043" (was in amp)
- "CAR3 in AH043" (was in amp)
- "CAR4#1 in PSB1C3" (was in cam)
- "DEL1 Ctrol no plasmid" (was in kan -> liquid clear, no growth?; pellet was also very small)

17:05 Centrifuged tubes 3 min 8000 rpm with Eppendorf 5424R (as advised by Tamannae). Discarded the supernatant and froze tubes at -70C, tower 33, new box "iGEM -15".

26.8.2015

WEDNESDAY, 8/26

Petra

Transforming yesterday's Gibson assembly reactions:
Transformed Gibson reactions for AtoB and CenA constructs to TOP10 cells. Made 2 transformations/reaction: used 4 ul Gibson reaction mix for each transformation. Followed the protocol. Plated each reaction (250 ul) on chloramphenicol plates.

Running SDS-PAGE for CAR samples:
Spinned the samples (made yesterday) for 1 min, 14000 rpm. Used the gel made yesterday. The other gel was left to the fridge and can be used later. Pipetted the samples (30 µl each) to the wells. Used 7 ul Bio-Rad Precision Plus Protein Dual color standard.
Pipetting order:

1. Marker
2. CAR in pSB1C3 gibson
3. CAR gibson 2 (in AH043)
4. CAR gibson 3 (in AH043)
5. CAR gibson 4 (in AH043)
6. CAR gibson 7 (in AH043)
7. Control: DEL1 cells without plasmid

Ran the gel first in 90 V and rinsed to 120 V when the marker was separated a bit. Total time ran: 1 h.

Staining the gel:
Stained the gel in Coomassie Brilliant Blue for 30 min with shaking. Let the gel sit in destaining buffer o/n.

Analytical restriction for CAR in AH043 backbone:
Restricted CAR with BamHI and SalI separately to analyze whether the restricted parts are right-sized. Used Thermo Fischer fast digest-enzymes (0,5 µl each) and Thermo Fisher's buffer (2,5 µl). Restriction with SalI produces 6342bp & 2583bp pieces and restriction with BamHI 5569bp, 2520bp, 698bp ja 138bp pieces. Used NeBuffer 3.1 for both of the enzymes.

Used 250 ng each CAR construct:

- CAR gibson 2: 2,56 µl, water 19,44 µl
- CAR gibson 3: 2,24 µl, water 19,76 µl
- CAR gibson 4: 2,1 µl, water 19,9 µl
- CAR gibson 7: 2,66 µl, water 19,34 µl

Incubated in 37 C for 1h. Started incubation at 13.00. Inactivated in 80 C for 5min.

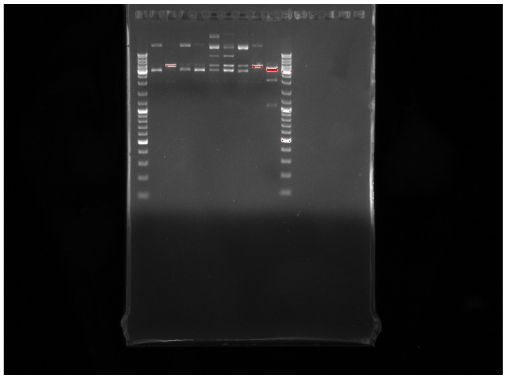
Restricted AH009 (plasmid, not linear) backbone with XbaI and SpeI. Used CutSmart buffer. Used 500 ng plasmid DNA = 9,68 µl and 11,82 ul water. Followed the protocol. Incubated in 37 C for 1h 30 min and inactivated in 80 C for 20 min.

Checking the restrictions on gel:
Made a 1,3 % agarose gel with ETBR. Ran 5 µl samples of CAR analytic restrictions and 5 µl sample of AH009 restriction (with 1 ul LD) on gel. Didn't use LD for CAR samples, because the buffer used before already contained it. Used GeneRuler ladder. Pipetting order:

1. Ladder
2. CAR2 BamhI
3. CAR2 SalI
4. CAR3 BamhI
5. CAR3 SalI
6. CAR4 BamhI
7. CAR4 SalI
8. CAR7 BamhI
9. CAR7 SalI
10. AH009
11. Ladder

Ran the gel for 40 min, 120 V.

Geldoc_2015-08-26_15hr_59minCAR_in_AH043_analytic_restriction.jpg



According to the gel picture restriction for AH009 was successful, but some of the DNA hasn't been restricted at all.

Anna doing gibson for AtoB & Cellulose constructs.
Cellulose:
CenA part1: 0,48µl
CenA part2: 0,36µl
BglX: 0,22µl
Restricted AH009: 3,2µl
Gibson Master Mix: 4,26µl

AtoB:
AtoB part 1: 0,38µl
AtoB part2: 0,22µl
AtoB part3: 0,2µl
AtoB part4: 0,84µl
Restricted AH009: 3,2µl
Gibson MasterMix: 4,84µl

Both incubated in +50C for 60min

Transformed 2µl and 4µl of reactions into TOP10 competent cells. Followed the transformation protocol, except on-ice incubation after heatshock lasted for 3 minutes. Rest of the gibson reaction mix stored in -20C.

Plated all of 2µl transformants on Cm plates and 50µl & 150µl of 4µl transformants on Cm plates.

Did a restriction of AH043 with X & S following mostly the protocol. However, restricted 500 ng of DNA and the incubation was 1,5 h.

Did gibson for Car Amph. In the reaction mix, there was:
Car part 1 1,2 ul
Car part 2 0,3 ul
Car Amph part 3 0,9 ul
Car Amph part 4 0,4 ul
AH043 6,3 ul
H20 1,8 ul
Buffer 9,1 ul

Transformed gibson of Car Amph construct 5 ul to NEB CHEM cells following the protocol. Plated 50 ul and 150 ul to AMP plates.

Did minipreps of AH045+GFP folowing the kit protocol.
NanoDrop results:

Table2

Sample	DNA (ng/ul)	A260/A280
AH045+GFP 1	35,1	1,83
AH045+GFP 2	45,4	1,81
AH045+GFP 3	21,0	1,79
AH045+GFP 4	38,9	1,84
AH045+GFP 5	36,0	1,84
AH045+GFP 6	21,9	1,90
AH045+GFP 7	20,7	1,86
AH045+GFP 8	34,5	1,85
AH045+GFP 9	46,4	1,83
AH045+GFP 10	30,5	1,85
AH045+GFP 11	69,9	1,81
AH045+GFP 12	28,6	1,84
AH045+GFP 13	43,2	1,82
AH045+GFP 14	25,1	1,86
AH045+GFP 15	57,9	1,86
AH045+GFP 16	31,0	1,86
AH045+GFP 17	14,7	1,88

Anna restricted Tamannae´s MiniPreps (AH045+GFP 1-17) with EcoRI & SpeI for gel analysis and possible ligation (if positive plasmids are found on gel). Used 3.1 buffer and followed the protocol. Incubation in +37C 90mins. Stored after inactivation in -20C.

Table1

Colony	DNA	H2O
1	7,1	14,4
2	5,5	16
3	11,9	9,6
4	6,4	15,1
5	6,9	14,6
6	11,4	10,1
7	12,1	9,4
8	7,2	14,3
9	5,4	16,1
10	8,2	13,3
11	3,6	17,9
12	8,7	12,8
13	5,8	15,7
14	10	11,5
15	4,3	17,2
16	8,1	13,4
17	17,7	4,4

27.8.2015

THURSDAY, 8/27

Petra

Checking yesterday's AtoB and CenA gibson transformants:
Gibson reactions made 25.8 with restricted *linearized* pSB1C3 backbone:

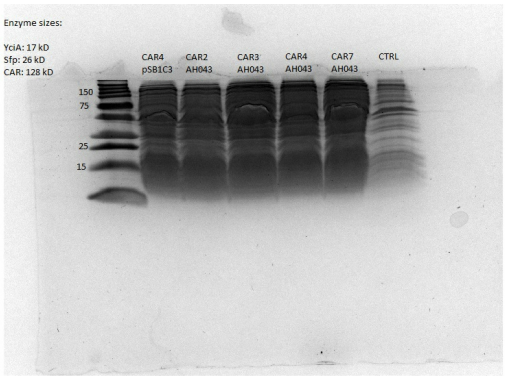
- No colonies on transformation plates.
- Our conclusion is that linearized backbone cut with EcoRI ansd PstI doesn't contain homologous sequence needed for Gibson assembly, because prefix and suffix are mostly cut away

Gibson reactions made 26.8 with AH009 backbone resticted with XbaI and SpeI:

- colonies on every AtoB and CenA plate
- Both colorless and reddish colonies, it's difficult to separate colorless colonies from those that will turn out red later
- Decided to choose colorless-looking colonies for further analysis

Took a picture of SDS-PAGE gel incubated in destaining buffer overnight:

SDS-page_Geldoc_2015-08-27_10hr_39minCAR.jpg



According to the gel picture bacteria that contain the inserted plasmid produce more enzymes than control bacteria. However it's impossible to say whether those encymes are YciA, Sfp and CAR. Decided to dilute the samples and run an another SDS-PAGE later.

Colony PCR fo AtoB and CenA

Chose 5 colonies from AtoB plates and 5 colonies from CenA plates to screen with colony PCR

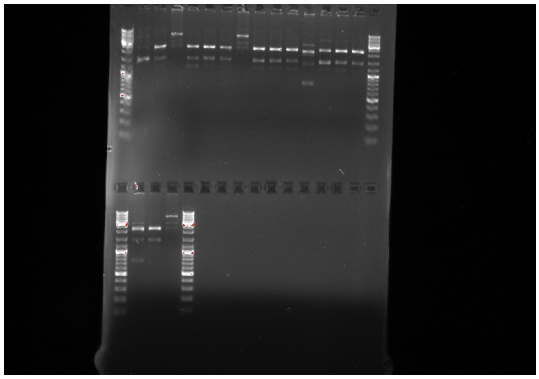
10x reaction mix without template DNA (230 µl)
125 ul NEB Q5 high fidelity mastermix
12,5 µl P001
12,5 µl P011
80 µl water

Pipetted 23 µl reaction mix to 10 PCR tubes. Added 2 ul template DNA to eas tube.

NEB Q5 colony PCR for CenA and AtoB from Gibson assembly: PCR program
98° *C - 30 s*
98° *C - 10 sec*
68° *C - 20 sec*
72° *C - 3,5 min*
72° *C - 2 min*
4° *C - forever*
Repeated underlined cycles 25 times

Made a 1,3 % agarose gel with EtBr. Run the yesterday's restrictions of AH045+GFP in the gel for 40 min with 100 V.
Pipeting order was:
I 1. ladder 2 ul 2.-15. AH045+GFP restrictions 1-14 6 ul 16. ladder 2 ul
II 1. ladder 2 ul 2.-4. AH045+GFP restrictions 15-17 6 ul 5. ladder 2 ul

Geldoc_2015-08-27_10hr_11min_restAH045GFP.jpg



The result: It seems that only colonies 11 and 15 have the correct insert (AH045+GFP, 820 bp).

Yesterday's transformation of gibson Car Amph was successful as there were 10 white colonies in the plates (most of the colonies were red). Therefore, did o/n cultures of Car Amph in 2 ml LB with 2 ul AMP (total: 10 tubes).

Did also preparations for colony PCR of Car Amph by making DNA templates from the colonies and making a screening plate (10 lines). The plate incubated o/n in 37 C and the DNA templates were strored in -20 C.

Restricted Amph + linker with X & P following the protocol. However, the incubation was 1,5 h. Stored the restriction in - 20 C.

28.8.2015

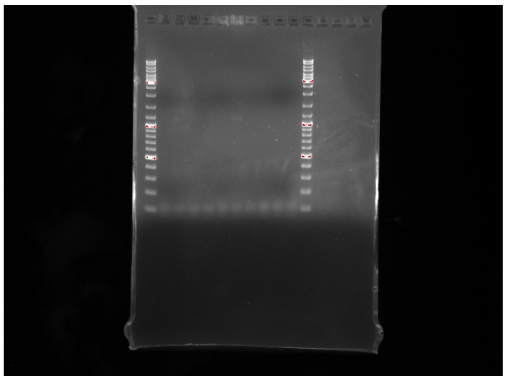
FRIDAY, 8/28

Petra

Made a 1,2 % agarose gel with ETBR. Ran 5 ul samples of yesterday's AtoB and CenA colony PCR reactions on gel with 1 ul LD. Used GeneRuler ladder. Ran the gel for 55 min, 100 V. Pipetting order:

1. Ladder
2. AtoB gibson colony 1
3. AtoB gibson colony 2
4. AtoB gibson colony 3
5. AtoB gibson colony 4
6. AtoB gibson colony 5
7. CenA gibson colony 1
8. CenA gibson colony 2
9. CenA gibson colony 3
10. CenA gibson colony 4
11. CenA gibson colony 5
12. Ladder

Geldoc_2015-08-28_14hr_14min_cpcr_atob_cena.jpg



According to the gel picture colony PCR for AtoB and CenA constructs was unsuccesful. Decided to do a restriction analysis for the plasmids that the colonies contain later, becase the colonies haven't turned red. Discarded the samples.

Making competent cells:
Made new TOP10 competent cells. Used o/n culture made on Wednesday 26.8. Pipetted 500 µl culture and 50 ml fresh LB to 500 ml Erlenmeyer flask . Added 25 ul streptomycin (final concentration in the culture: 25 µg/ml). Followed the protocol.

Making chloramphenicol plates:
Made more chloramphenicol plates. Followed the protocol.

Did colony PCR of Car Amph using the KAPA kit. Because the screening plate showed that one of colonies was red (10), used 9 different DNA templates (so 9 cPCR reactions), which were made yesterday.
Reaction mix of Car Amph (25 ul):
15,25 ul H20
5 ul Buffer
0,75 ul NTP
0,75 ul 10 uM p001
0,75 ul 10 uM p011
0,5 ul KAPA HotStart
2 ul DNA template from a colony of Car Amph

PCR program:
95° *C - 3 min*
98° *C - 20 sec*
64° *C - 15 sec*
72° C - 280 sec

72° C - 6 min
4° C - forever
Repeated underlined cycles 25 times

Restricted linear pSB1A3 and pSB1T3 with E & P following the protocol. However, the incubation was 1,5 h.

Ligated twice the restrictions of AH045+GFP 11/15, pSB1A3/pSB1T3 and Amph + linker. One set of ligations was done following the T4 ligase protocol's suggestion of molar ratio 3:1 of inserts (AH045+GFP 11/15 3,5 ul with PSB1A3 & 3 ul with pSB1T3; Amph+linker 3,2 ul with PSB1A3 & 2,9 ul with pSB1T3) and vectors (pSB1A3/pSB1T3 3 ul). The other set was done so that the volume of the inserts (4 ul of the both) was the twice as more than a vector's (pSB1A3 2 ul).

Transformed the ligations to Top10 CHEM competent cells following the protocol. Plated 50 ul and 150 ul from each tube (total: 6 tubes) to AMP and TC plates appropriately. Incubated in 37 C o/n.

Miniprepped o/n cultures of Car Amph following the kit protocol. However, the culture of Car Amph 9 was red so it wasn't miniprepped.
NanoDrop results:

Table1

Sample	DNA (ng/ul)	A260/A280
Car Amph 1	89,4	1,84
Car Amph 2	69,1	1,87
Car Amph 3	77,6	1,85
Car Amph 4	79,4	1,87
Car Amph 5	83,1	1,85
Car Amph 6	76,1	1,87
Car Amph 7	64,8	1,86
Car Amph 8	73,5	1,86
Car Amph 10	70,4	1,86